

### REMARKS

Applicant submits the present amendment to claims 1 and 2. Support for the amendment to claim 1 is found on, e.g., page 7 of the specification. No new matter is added by the amendment.

The Office objects to claim 2 for redundancy. Claim 2 has been amended according to the Examiner's suggestion. This objection can be withdrawn.

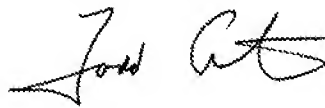
The Office rejects pending claims 1 and 2 for obviousness over Pittenger et al. (WO 99/03973; hereinafter "Pittenger") in view of Bruder et al. (WO 97/39104; hereinafter "Bruder") or Abatangelo et al. (U.S. Patent No. 6,482,231; hereinafter "Abatangelo"), as evidenced by Ha et al. (U.S. Patent Application Publication No. 2005/0118714; hereinafter "Ha"). None of the publications cited in the Office Action, Pittenger, Bruder, Abatangelo, or Erices, teaches or suggests, let alone demonstrates, use of umbilical cord blood-derived USSCs to treat cardiac muscle disease. Furthermore, as acknowledged by the Examiner during a telephonic communication dated October 15, 2008, mesenchymal stem cells (MSCs) from bone marrow are not the same as human unrestricted somatic stem cells (USSCs) isolated from umbilical cord; the Examiner acknowledged that Kern et al. (Stem Cells 24:1294-1301, 2006; a copy of which is provided herewith) shows this difference. This rejection can be withdrawn.

### CONCLUSION

Present claims 1 and 2 are in condition for allowance, and such action is respectfully requested.

A petition to extend the period for replying for one month, to and including November 1, 2008, is submitted herewith. Applicants authorize the Office to deduct the fee required by 37 C.F.R. § 1.17(a) for the petition from Deposit Account No. 03-2095. If there are any charges or any credits, please apply them to Deposit Account No. 03-2095.

Respectfully submitted,



Todd Armstrong, Ph.D.  
Reg. No. 54,590

Date: 20 October 2008

for Paul T. Clark  
Reg. No. 30,162

Clark & Elbing LLP  
101 Federal Street  
Boston, MA 02110  
Telephone: 617-428-0200  
Facsimile: 617-428-7045

## Comparative Analysis of Mesenchymal Stem Cells from Bone Marrow, Umbilical Cord Blood, or Adipose Tissue

SUSANNE KERN,<sup>a</sup> HERMANN EICHLER,<sup>a</sup> JOHANNES STOEVE,<sup>b</sup> HARALD KLÜTER,<sup>a</sup> KAREN BIEBACK<sup>a</sup>

<sup>a</sup>Institute of Transfusion Medicine and Immunology, German Red Cross Blood Service of Baden-Württemberg-Hessen, Mannheim, Germany; <sup>b</sup>Department of Orthopaedic Surgery of the University Hospital Mannheim, University of Heidelberg, Faculty of Clinical Medicine, Mannheim, Germany

**Key Words.** Mesenchymal stem cells • Bone marrow • Umbilical cord blood • Adipose tissue • Comparative analysis  
Multilineage differentiation

### ABSTRACT

Mesenchymal stem cells (MSCs) represent a promising tool for new clinical concepts in supporting cellular therapy. Bone marrow (BM) was the first source reported to contain MSCs. However, for clinical use, BM may be detrimental due to the highly invasive donation procedure and the decline in MSC number and differentiation potential with increasing age. More recently, umbilical cord blood (UCB), attainable by a less invasive method, was introduced as an alternative source for MSCs. Another promising source is adipose tissue (AT). We compared MSCs derived from these sources regarding morphology, the success rate of isolating MSCs, colony frequency, expansion potential, multiple differentiation capacity, and immune phenotype. No significant differences concerning the morphology and immune pheno-

type of the MSCs derived from these sources were obvious. Differences could be observed concerning the success rate of isolating MSCs, which was 100% for BM and AT, but only 63% for UCB. The colony frequency was lowest in UCB, whereas it was highest in AT. However, UCB-MSCs could be cultured longest and showed the highest proliferation capacity, whereas BM-MSCs possessed the shortest culture period and the lowest proliferation capacity. Most strikingly, UCB-MSCs showed no adipogenic differentiation capacity, in contrast to BM- and AT-MSCs. Both UCB and AT are attractive alternatives to BM in isolating MSC: AT as it contains MSCs at the highest frequency and UCB as it seems to be expandable to higher numbers. *STEM CELLS* 2006;24:1294–1301

### INTRODUCTION

Mesenchymal stem cells (MSCs) found in many adult tissues are an attractive stem cell source for the regeneration of damaged tissues in clinical applications because they are characterized as undifferentiated cells, able to self-renew with a high proliferative capacity, and possess a mesodermal differentiation potential [1].

Although bone marrow (BM) has been the main source for the isolation of multipotent MSCs, the harvest of BM is a highly invasive procedure and the number, differentiation potential, and maximal life span of MSCs from BM decline with increasing age [2–4]. Therefore, alternative sources from which to isolate MSCs are subject to intensive investigation.

One alternative source is umbilical cord blood (UCB), which can be obtained by a less invasive method, without harm for the mother or the infant [5]. However, controversy still exists whether full-term UCB can serve as a source for isolating multipotent MSCs: although some groups did not succeed in

isolating MSCs [6, 7], we and other groups succeeded in isolating MSCs from full-term UCB [8–12].

Adipose tissue (AT) is another alternative source that can be obtained by a less invasive method and in larger quantities than BM. It has been demonstrated that AT contains stem cells similar to BM-MSCs, which are termed processed lipoaspirate (PLA) cells [13]. These cells can be isolated from cosmetic liposuctions in large numbers and grown easily under standard tissue culture conditions [13]. The multilineage differentiation capacity of PLA cells has been confirmed [13].

As BM-MSCs are best characterized, we asked whether MSCs derived from other sources share the characteristics of BM-MSCs. The aim of our study was to compare MSCs isolated from the three sources under identical in vitro conditions with respect to their morphology, frequency of colonies, expansion characteristics, multilineage differentiation capacity, immunophenotype, and success rate of isolating the cells.

Correspondence: Karen Bieback, Ph.D., Institute of Transfusion Medicine and Immunology, German Red Cross Blood Service of Baden-Württemberg – Hessen, University of Heidelberg, Faculty of Clinical Medicine Mannheim, Friedrich-Ebert-Straße 107, D-68167 Mannheim, Germany. Telephone: +49-621-3706-8216; Fax: +49-621-3706-851; e-mail: k.bieback@itima.blutspende.de Received July 28, 2005; accepted for publication December 21, 2005; first published in *STEM CELLS EXPRESS* January 12, 2006. ©AlphaMed Press 1066-5099/2006/\$20.00/0 doi: 10.1634/stemcells.2005-0342

## MATERIALS AND METHODS

### Collection of BM

BM aspirates of 18 patients ranging in age from 68–84 years were obtained from the femoral shaft undergoing total hip replacement at the orthopedic department of the University Hospital Mannheim. Additional BM aspirates were obtained from three patients ranging in age from 44–71 years by puncturing the iliac crest. The BM aspirates were received in accordance with the ethical standards of the local ethical committee.

### Isolation and Culture of Mononuclear Cells from BM

The aspirates were diluted 1:5 with 2 mM ethylenediaminetetraacetic acid (EDTA)-phosphate-buffered saline (PBS) (Merck & Co., Whitehouse Station, NY, <http://www.merck.com>; Nexcel, Baxter, Unterschleissheim, Germany, <http://www.baxter.de>). The MNC fraction was isolated by density gradient centrifugation at 435g for 30 minutes at room temperature using Ficoll-Hypaque-Plus solution (GE Healthcare BioSciences Corp., Piscataway, NJ, <http://www.gehealthcare.com>) and seeded at a density of  $1 \times 10^6$  cells per  $\text{cm}^2$  into T75 or T175 cell culture flasks (Nunc, Rochester, NY, <http://www.nuncbrand.com>; Greiner Bio-One, Frickenhausen, Germany, <http://www.gbo.com>). The first change of medium was accomplished within 3 days after isolation. The resulting fibroblastoid adherent cells were termed BM-derived fibroblastoid adherent cells (BM-FACs) and were cultivated at 37°C at a humidified atmosphere containing 5%  $\text{CO}_2$ . The expansion medium consisted of either mesenchymal stem cell growth medium Bullet-Kit (MSCGM; Cambrex, Walkersville, MD, <http://www.cambrex.com>) or Dulbecco's modified Eagle's medium-low glucose (DMEM-lg) containing 10% mesenchymal stem cell growth supplements (MSCGS) (Cambrex). FACs were maintained in MSCGM or DMEM-lg + 10% MSCGS until they reached 70% to 90% confluency. Cells were harvested at subconfluence using Trypsin (PromoCell, Heidelberg, Germany, <http://www.promocell.com>). Cells at the second passage and thereafter were replated at a mean density of  $1.3 \pm 0.7 \times 10^3/\text{cm}^2$ .

Generation of single separated, fibroblastoid colonies termed fibroblastoid colony-forming units (CFU-F) was achieved by initially seeding the MNCs at a low density ( $1 \times 10^3$  to  $1 \times 10^4$  cells per  $\text{cm}^2$ ). CFU-F were selected and isolated either using Trypsin (PromoCell) or by scraping them off from the surface of the culture plate with the tip of a pipette. Subcultivation was performed as described for the BM-FACs.

### Collection of UCB

UCB units ( $n = 59$ ) were collected from the unborn placenta of full-term deliveries in a multiple bag system containing 17 ml of citrate phosphate dextrose buffer (Cord Blood Collection System; Eltest, Bonn, Germany) [14, 15] and processed within 24 hours of collection. The collection was performed in accordance with the ethical standards of the local ethical committee.

### Isolation and Culture of MNC from UCB

The isolation of MSCs was performed as described for BM with a few exceptions. Prior to the isolation of MNC, the anticoagulated cord blood was diluted 1:1 with 2 mM EDTA-PBS. The MNC fraction was initially seeded at a density of  $1 \times 10^6$

MNC/ $\text{cm}^2$  into fetal calf serum (FCS)-precoated culture plates (FCS batches S0113/1038E and S0113/892E; Biochrom, Berlin, Germany, <http://www.biochrom.de>) (Falcon, Becton, Dickinson and Company, Franklin Lakes, NJ, <http://www.bd.com>) [8]. Nonadherent cells were removed 12–18 hours after initial plating. The same culture conditions and media were applied as described for BM-FACs. Adherent fibroblastoid cells only appeared as CFU-F and were harvested at subconfluence using Trypsin (PromoCell). Cells at the second passage and thereafter were replated at a mean density of  $3.5 \pm 4.8 \times 10^3/\text{cm}^2$ .

### Collection of AT

AT was obtained from 18 donors ranging in age from 26–57 years undergoing liposuction procedures. Lipoaspirates were obtained in accordance with the ethical standards of the local ethical committee.

### Isolation and Culture of PLA cells from AT

The raw lipoaspirate (50–100 ml) was processed as described previously [13]. To isolate the stromal vascular fraction (SVF), lipoaspirates were washed intensely with PBS. Thereafter the lipoaspirates were digested with an equal volume of 0.075% collagenase type I (Sigma-Aldrich, St. Louis, <http://www.sigmaaldrich.com>) for 30–60 minutes at 37°C with gentle agitation. The activity of the collagenase was neutralized with DMEM-lg containing 10% FCS. To obtain the high-density SVF pellet, the digested lipoaspirate was centrifuged at 1,200g for 10 minutes. The pellet was resuspended in MSCGM or DMEM-lg containing 10% MSCGS and filtered through a 100  $\mu\text{m}$  nylon cell strainer (Falcon). The filtered cells were centrifuged at 1,200g for 10 minutes. The resuspended SVF cells were plated at a density of  $1 \times 10^6/\text{cm}^2$  into T75 or T175 culture flasks. Nonadherent cells were removed 12–18 hours after initial plating by intensely washing the plates. The resulting fibroblastoid adherent cells were termed AT-derived fibroblastoid adherent cells (AT-FACs). AT-FACs were cultivated under the same conditions as described for BM-FACs. AT-FACs were harvested at subconfluence using Trypsin (PromoCell). Cells at the second passage and thereafter were replated at a mean density of  $1.8 \pm 3.1 \times 10^3/\text{cm}^2$ .

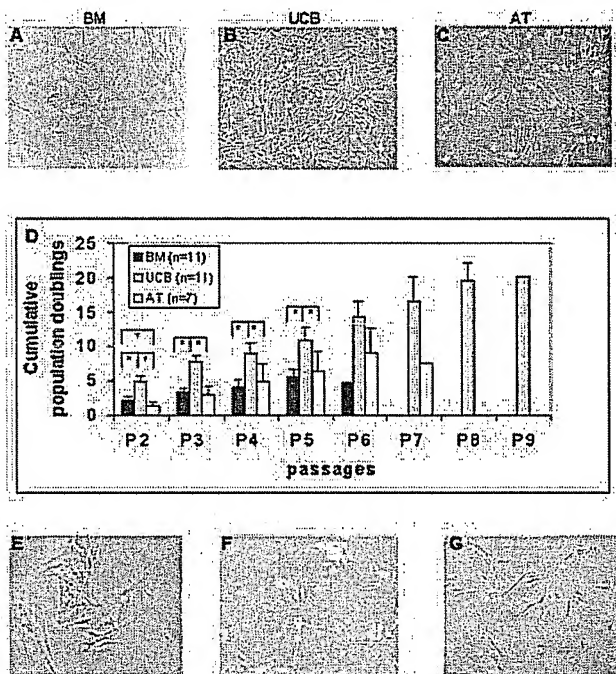
The generation of single separated CFU-F was achieved by initially seeding the SVF cells at a low density ( $1 \times 10^2$  to  $1 \times 10^3$  cells per  $\text{cm}^2$ ). CFU-F were selected and isolated as described for BM-CFU-F. Subcultivation of the cells was performed as described for the AT-FACs.

### Primary Dermal Fibroblasts as Controls

Primary normal dermal fibroblasts (PromoCell) served as negative controls in the differentiation studies. The cells were cultured in 10% supplemented fibroblast growth medium (PromoCell).

### Expansion Characteristics

Fibroblastoid cells from BM, UCB, and AT were harvested at subconfluence as described in the previous paragraphs. The cultures were abandoned as soon as they showed a senescent phenotype defined by two criteria: 1) when at least 90% of the cells adopted an altered morphology resembling senescent cells within 3–4 weeks after subcultivation (Fig. 1E–1G); and 2) when they ceased proliferation. The senescence ratio was determined up to passage 2 calculating the ratio of samples



**Figure 1.** Morphology of adherent cells after initial plating and at the senescent phase. (A–C): Fibroblastoid adherent cells after initial plating derived from the fibroblastoid adherent cell (FAC) monolayer of BM at day 14 (A), from a UCB-fibroblastoid colony-forming units (CFU-F) at day 16 (B), and from the FAC monolayer of AT at day 8 (C). (E–G): Senescent cells from the FAC monolayer of BM at passage 2 (E), from an UCB-CFU-F at passage 4 (F), and from the FAC monolayer of AT at passage 6 (G) were identified by an altered morphology. All representative examples are shown at a magnification of  $\times 100$ . (D): Mean values of the cumulative population doublings. Population doublings were determined at each subcultivation; mean values of BM-mesenchymal stem cells (MSCs) are shown in black; mean values of UCB-MSCs are shown in gray; mean values of AT-MSCs are shown by open bars. The starting sample numbers at passage 2 are given in the diagram. As samples underwent senescence, the number of samples went down at proceeding passages; the number of samples at distinct passages gave rise to the following mean values. BM: p3,  $n = 9$ ; p4 and p5,  $n = 7$ ; p6,  $n = 1$ . UCB: p4,  $n = 9$ ; p5,  $n = 7$ ; p6–8,  $n = 4$ ; p9,  $n = 1$ . AT: p3,  $n = 6$ ; p4,  $n = 5$ ; p5 and p6,  $n = 2$ ; p7,  $n = 1$ . \*,  $p < .05$ . Abbreviations: AT, adipose tissue; BM, bone marrow; P, passage; UCB, umbilical cord blood.

adopting a senescent phenotype to the total number of samples cultured. Cumulative population doublings were calculated using the formula  $x = [\log_{10}(N_H) - \log_{10}(N_I)] / \log_{10}(2)$  [16], where  $N_I$  is the inoculum cell number and  $N_H$  the cell harvest number. To yield the cumulated doubling level, the population doubling for each passage was calculated and then added to the population doubling levels of the previous passages. As the cell number of fibroblastoid cells of all three tissues could be determined for the first time at passage 1, the cumulative doubling number was first calculated for passage 2.

### In Vitro Differentiation

Primary normal human dermal fibroblasts (PromoCell) served as negative controls in all three differentiation studies.

### Osteogenic Differentiation

Differentiation of each sample was performed at defined passages as follows: BM-FACs, passages 0–5 ( $n = 7$ ); BM-CFU-F,

passages 2–6 ( $n = 14$ ); UCB-CFU-F, passages 1–7 ( $n = 8$ ); AT-FACs, passages 0–5 ( $n = 14$ ); AT-CFU-F, passages 2–6 ( $n = 28$ ). To promote osteogenic differentiation, the cells were seeded at a density of  $3.1 \times 10^3$  cells per  $\text{cm}^2$  into eight-chamber-slides (Nunc) and cultured in MSCGM or DMEM-Ig + 10% MSCGS until they reached 70%–80% confluence. As soon as subconfluence was reached, osteogenic differentiation of the cells was induced by feeding them for 2.5 weeks, twice a week, with osteogenic induction medium consisting of 100 nM dexamethasone, 10 mM  $\beta$ -glycerophosphate, 0.2 mM ascorbate, and 10% FCS in osteogenic basal medium (Cambrex). For the negative control, the cells were kept in MSCGM or DMEM-Ig + 10% MSCGS. Osteogenic differentiation was confirmed by the increase of alkaline phosphatase (AP) expression by histochemical staining following the manufacturer's instructions (leukocyte alkaline phosphatase kit 85L-3R; Sigma-Aldrich). Later differentiation stages were detected by the von Kossa stain, demonstrating the deposition of a hydroxyapatite matrix. The von Kossa stain was performed according to the protocol described previously [17] with a few modifications. The cells were fixed with 10% formalin (Sigma-Aldrich) for 15 minutes at room temperature and stained for 10–15 minutes with 5% silver nitrate (Sigma-Aldrich). The stain was developed incubating the cells in 1% pyrogallol (Merck) and then fixed with 5% sodium thiosulfate (Sigma-Aldrich) for 5 minutes.

### Adipogenic Differentiation

Differentiation of each sample was performed at defined passages as follows: BM-FACs, passages 0–5 ( $n = 9$ ); BM-CFU-F, passages 2–6 ( $n = 14$ ); UCB-CFU-F, passages 1–7 ( $n = 11$ ); AT-FACs, passages 0–5 ( $n = 16$ ); AT-CFU-F, passages 2–6 ( $n = 28$ ). To induce adipogenic differentiation, the cells were seeded at a density of  $2.1 \times 10^4$  cells per  $\text{cm}^2$  into eight-chamber slides (Nunc) and cultured in MSCGM or DMEM-Ig + 10 MSCGS until reaching 100% confluence or postconfluence. Then, the cells were induced by three cycles of induction/maintenance [1] using adipogenic induction medium consisting of 1 mM dexamethasone, 0.5 mM 3-isobutyl-1-methyl-xanthine, 10  $\mu\text{g}/\text{ml}$  recombinant human insulin, 100 mM indomethacin, and 10% FCS (Cambrex) and using adipogenic maintenance medium consisting solely of 10  $\mu\text{g}/\text{ml}$  recombinant human insulin and 10% FCS (Cambrex). After completing the three cycles of induction and maintenance, the induced cells were incubated for another 7 days in adipogenic maintenance medium. The noninduced control cells were fed only with adipogenic maintenance medium. Adipogenic differentiation was confirmed by the formation of neutral lipid-vacuoles stainable with Oil Red O (Sigma-Aldrich). For the Oil Red O stain, cells were fixed with 10% formalin (Sigma-Aldrich), washed, and stained with a working solution of 0.18% Oil Red O for 5 minutes. The nuclei were counterstained with Mayer's hematoxylin solution (Sigma-Aldrich).

### Chondrogenic Differentiation

Differentiation of each sample and CFU-F was performed at defined passages as follows: BM-FACs, passages 0–5 ( $n = 8$ ); BM-CFU-F, passages 2–6 ( $n = 14$ ); UCB-CFU-F, passages 1–7 ( $n = 15$ ); AT-FACs, passages 0–5 ( $n = 18$ ); AT-CFU-F, passages 2–6 ( $n = 28$ ). For chondrogenic differentiation, cells were cultured in a micromass culture. Therefore  $2.5 \times 10^5$  cells

were centrifuged in a 15-ml polypropylene tube (Greiner) at 150g to form a pellet. Without disturbing the pellet, the cells were cultured for 4 weeks in 0.5 ml of complete chondrogenic differentiation medium (Cambrex) including 10 ng/ml TGF- $\beta$ -3 (Strathmann Biotec AG, Hamburg, Germany, <http://www.strathmann-biotec-ag.de>). Cells were fed twice a week. After the culture period, cryosections were analyzed by Safranin O staining. For the staining, the sections were fixed with ice-cold acetone (Sigma-Aldrich) and stained with 0.1% aqueous Safranin O for 5 minutes and the nuclei were counterstained with Weigert's iron hematoxylin (Sigma-Aldrich).

### Immunophenotypic Analyses

For further characterization, cell surface antigen phenotyping was performed on BM-MSCs at passages 0–7, of UCB-MSCs at passages 3–7, and of AT-MSCs at passages 1–5. The following cell-surface epitopes were marked with anti-human antibodies: CD14-fluorescein isothiocyanate (FITC), CD34-phycoerythrin (PE), CD73-PE, CD90-Cy5 (Becton Dickinson), CD29-PE, CD44-FITC, CD45-Peridium-chlorophyll protein complex (PerCP), HLA-class I-FITC, HLA-class II-FITC (Beckman Coulter, Fullerton, CA, <http://www.beckmancoulter.com>), CD133-PE (Miltenyi Biotec, Bergisch Gladbach, Germany, <http://www.miltenyibiotec.com>), CD105-FITC, and CD106-PE (Immunokontakt; AMS Biotechnology, Wiesbaden, Germany, <http://www.immunok.com>). Mouse isotype antibodies served as control (Becton Dickinson; Beckman Coulter). 10,000 labeled cells were acquired and analyzed using a FACScan flow cytometer running CellQuest software (Becton Dickinson).

### Statistical Analysis

Data are presented as mean  $\pm$  standard deviation. A two-sided, nonpaired *t* test was used to analyze the flow cytometry and the cumulative doubling data. A Mann-Whitney *U* test was used to compare the isolation efficacy of CFU-F derived from UCB, BM, and AT. A  $\chi^2$  test was applied to compare the differentiation capacities of cells isolated from the different tissues and ratios of samples with a senescent phenotype. Differences were considered significant at  $p < .05$ . The SPSS software package (version 12.0; SPSS Inc., Chicago, <http://www.spss.com>) was used for the statistical tests.

## RESULTS

### Isolation of Adherent Cells of Fibroblastoid Morphology from BM, UCB, and AT

At an initial plating density of  $1 \times 10^6$  cells per  $\text{cm}^2$ , both BM- and AT-derived fibroblastoid cells formed a monolayer 4–5 days after initial plating and were termed FACs. In contrast to BM or AT, UCB-derived fibroblastoid cells formed CFU-F and could be detected first 2–4 weeks after plating when applying the same initial plating density. In contrast to UCB, for the generation of CFU-F from BM and AT a lower initial plating density was necessary. The number of CFU-F calculated at the basis of  $1 \times 10^6$  initially plated cells was highest for AT ( $557 \pm 673$ ), followed by BM ( $83 \pm 61$ ); it was lowest for UCB ( $0.002 \pm 0.004$ ) ( $p < .001$ ).

The success rate of isolating FACs and CFU-F from both BM ( $n = 21$ ) and AT ( $n = 18$ ) was 100%. In contrast, the success rate in UCB ( $n = 59$ ) was only 29% from all units

**Table 1.** Senescence ratio and maximal expansion potential of mesenchymal stem cells (MSCs) derived from BM, UCB, and AT

|                  | Senescence ratio up to passage 2 | Maximal passage           |
|------------------|----------------------------------|---------------------------|
| BM ( $n = 21$ )  | 23.6%                            | P 7 (9.5%)                |
| UCB ( $n = 26$ ) | 34.6%                            | >P 10 <sup>a</sup> (3.9%) |
| AT ( $n = 18$ )  | 5.6%                             | P 8 (5.6%)                |
| $p = .02^b$      |                                  |                           |

The data represent the ratios of samples undergoing early senescence up to the second passage and samples that could be cultured up to the indicated maximal passage until undergoing senescence. Senescence ratios were determined by calculating the number of samples adopting an altered morphology (Fig. 1E–1G) and ceasing proliferation to the total number of samples cultured.

<sup>a</sup>The culture could be cultured at minimum up to the indicated passage, since it had to be discarded due to contaminations.

<sup>b</sup>Significant differences of the senescence ratio were observed between AT and UCB.

Abbreviations: AT, adipose tissue; BM, bone marrow; UCB, umbilical cord blood.

processed. The rate could be enhanced to 63% by FCS precoat-ing and by taking into account only units of optimal quality [8]. However, no differences concerning the morphology of the adherent cells derived from the three tissues were obvious (Fig. 1A–1C).

### Expansion Characteristics

Analysis of proliferation capacities of the MSCs derived from the three sources showed that BM-MSCs possessed the lowest population doubling numbers through passages 4–6, followed by AT-MSCs after passage 3 (Fig. 1D). However, UCB-MSCs displayed the highest doubling numbers in all passages analyzed.

During in vitro culture, MSCs possess a limited life span and finally undergo replicative senescence indicated by loss of proliferation and altered morphology. Analysis of senescence ratios and maximal expansion potentials of BM-MSCs ( $n = 21$  donors), of UCB-MSCs ( $n = 26$  CFU-F, 16 UCB units), and of AT-MSCs ( $n = 18$  donors) demonstrated that UCB contained the highest ratio of MSCs undergoing senescence within early passages, followed by BM-MSCs (Fig. 1E–1G; Table 1). The lowest senescence ratio within early passages displayed AT-MSCs. Despite many colonies reaching senescence at early stages, other UCB-MSCs could be cultured longest, followed by AT-MSCs, whereas BM-MSCs showed the shortest culture period (Table 1).

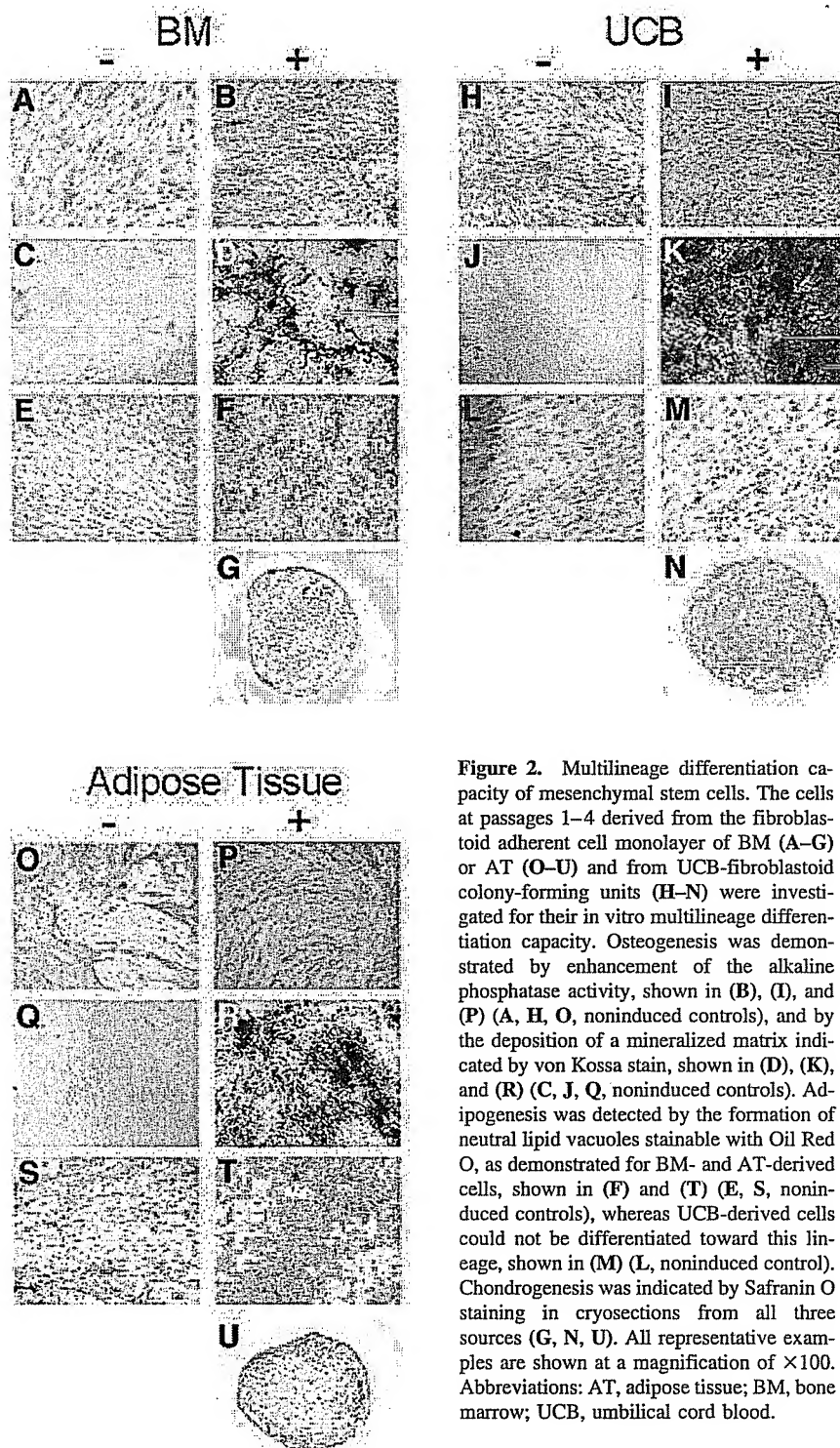
### Multilineage Differentiation Potential

To demonstrate the isolation of MSCs and to investigate their differentiation potential, the fibroblastoid cells derived from the three sources were directed toward the osteogenic, adipogenic, and chondrogenic lineages at the respective passages. Fibroblasts served as negative controls.

### Osteogenic Differentiation Capacity

Osteogenic differentiation was confirmed by the detection of an osteogenic phenotype consisting of an increased expression of AP and by the deposition of a silver stained mineralized matrix.





**Figure 2.** Multilineage differentiation capacity of mesenchymal stem cells. The cells at passages 1–4 derived from the fibroblastoid adherent cell monolayer of BM (A–G) or AT (O–U) and from UCB-fibroblastoid colony-forming units (H–N) were investigated for their in vitro multilineage differentiation capacity. Osteogenesis was demonstrated by enhancement of the alkaline phosphatase activity, shown in (B), (I), and (P) (A, H, O, noninduced controls), and by the deposition of a mineralized matrix indicated by von Kossa stain, shown in (D), (K), and (R) (C, J, Q, noninduced controls). Adipogenesis was detected by the formation of neutral lipid vacuoles stainable with Oil Red O, as demonstrated for BM- and AT-derived cells, shown in (F) and (T) (E, S, noninduced controls), whereas UCB-derived cells could not be differentiated toward this lineage, shown in (M) (L, noninduced control). Chondrogenesis was indicated by Safranin O staining in cryosections from all three sources (G, N, U). All representative examples are shown at a magnification of  $\times 100$ . Abbreviations: AT, adipose tissue; BM, bone marrow; UCB, umbilical cord blood.

After 2.5 weeks of induction, no significant differences in the osteogenic differentiation capacity were detected ( $p = .4$ ), since 100% (eight of eight) of the UCB samples, 71.4% (five of seven) of the BM samples, and 78.8% (11 of 14) of the AT samples showed an osteogenic phenotype (Fig. 2B, 2D, 2I, 2K, 2P, 2R). No distinct osteogenic phenotype was noticed for 28.6% (two of seven) of the BM samples or for 21.4% (3 of 14)

of the AT samples, since only an increased expression of AP and no deposition of a matrix was observed. No osteogenic phenotype was induced in the fibroblasts (not shown).

#### Adipogenic Differentiation Capacity

Adipogenic differentiation was demonstrated by the accumulation of neutral lipid vacuoles indicated by the Oil Red O stain.

After adipogenic induction, 100% (nine of nine) of the BM samples and 94% (15 of 16) of the AT samples possessed cells with an adipogenic phenotype (Fig. 2F, 2T). However, none of the UCB-derived CFU-F displayed an adipogenic phenotype under standard differentiation conditions. Even after applying a fortified differentiation protocol in which cells were induced for 5 weeks in adipogenic induction medium only, no adipocytes were detectable (Fig. 2M). Thus, a significantly higher number of BM and AT samples than that of UCB featured an adipogenic differentiation capacity ( $p < .01$ ). No adipogenic phenotype was induced in the fibroblasts (not shown).

### Chondrogenic Differentiation Capacity

Chondrogenic differentiation was confirmed by the formation of a sphere in the micromass culture and the secretion of cartilage-specific proteoglycans stainable with Safranin O. All samples tested, irrespective of their origin, as well as the fibroblasts, demonstrated a cartilage-like phenotype with chondrocyte-like lacunae (Fig. 2G, 2N, 2U; fibroblasts not shown).

### Comparison of Multilineage Differentiation Capacity

Comparing the multilineage differentiation capacity, we analyzed only those samples for which results of the three differentiation capacities were obtained. Differentiation capacities into all three lineages was demonstrated for 71.4% of BM and AT samples (five of seven; 10 of 14). All (six of six) UCB-CFU-F analyzed could only be directed toward two lineages, due to the nondetectable adipogenic differentiation capacity. A two-lineage differentiation capacity only was observed for significantly fewer BM and AT samples than for UCB samples (BM, 28.6% [two of seven]; AT, 28.6% [4 of 14];  $p = .008$ ); all BM and almost all AT samples showed an adipo-chondrogenic differentiation capacity. Only one AT sample demonstrated an osteo-chondrogenic differentiation capacity.

### Multilineage Differentiation Capacity of BM- and AT-CFU-F

Since UCB-MSCs displayed no adipogenic differentiation capacity, in contrast to AT- and BM-MSCs, we hypothesized that this might be related to the CFU-F-derived origin of UCB-MSCs. BM- and AT-FACs originated from a monolayer, which might contain a more heterogeneous mixture of precursor cells in contrast to CFU-F. Therefore, CFU-F were also generated from BM and AT and analyzed for their differentiation capacity. The differentiation capacity of CFU-F from both tissues was not restricted: a differentiation capacity toward all three lineages could be observed for 28.6% (4 of 14) of BM-CFU-F and for 89.3% (25 of 28) of the AT-CFU-F ( $p < .001$ ). A differentiation capacity toward two lineages only was noticed for 64.2% (9 of 14) of BM-CFU-F and for 7.1% (2 of 28) of AT-CFU-F ( $p < .001$ ): all of the AT-CFU-F and almost all of the BM-CFU-F showed an adipo-chondrogenic differentiation capacity, since one BM-CFU-F possessed an osteo-chondrogenic differentiation capacity. One BM- and AT-derived CFU-F could be differentiated toward only one, the chondrogenic lineage.

### Immunophenotypic Characterization

For further characterization of the MSCs, surface protein expression of MSCs of nine BM donors at passages 0–7, of 13 UCB-CFU-F (8 UCB units) at passages 3–5, and of nine AT

**Table 2.** Comparison of the expression of surface proteins of mesenchymal stem cells derived from BM, UCB, and AT as analyzed by flow cytometry

| Antibody           | BM (%)<br>(n = 9) | UCB (%)<br>(n = 10) | AT (%)<br>(n = 9) |
|--------------------|-------------------|---------------------|-------------------|
| CD44               | 97.5 ± 5.1        | 99.7 ± 0.5          | 99.8 ± 0.2        |
| CD73               | 90.0 ± 20.0       | 99.3 ± 1.3          | 99.6 ± 0.5        |
| CD90               | 99.1 ± 2.5        | 97.8 ± 7.1          | 99.9 ± 0.2        |
| CD14               | 1.2 ± 1.1         | 0.8 ± 0.9           | 2.4 ± 5.0         |
| CD34               | 1.6 ± 1.4         | 1.2 ± 1.5           | 5.0 ± 5.1         |
| CD45               | 5.2 ± 3.7         | 3.8 ± 3.6           | 3.8 ± 5.1         |
| CD105 <sup>a</sup> | 88.1 ± 7.4        | 72.4 ± 20.0         | 90.4 ± 5.9        |
| CD133              | 1.3 ± 1.1         | 2.9 ± 5.5           | 2.9 ± 3.5         |
| CD29               | 99.0 ± 1.5        | 99.8 ± 0.4          | 99.5 ± 1.3        |
| HLA I              | 95.2 ± 6.0        | 94.3 ± 6.8          | 98.8 ± 2.8        |
| CD106 <sup>b</sup> | 66.3 ± 22.7       | 70.0 ± 23.6         | 30.3 ± 18.6       |
| HLA II             | 4.2 ± 6.1         | 0.8 ± 1.2           | 4.4 ± 6.2         |
| CD144              | 4.5 ± 9.3         | 2.4 ± 4.0           | 2.4 ± 2.5         |

The table shows mean values of the percentage of positive cells ± standard deviation to the total number of cells analyzed.

<sup>a</sup>Significant differences were observed between UCB compared with BM and AT ( $p < .05$ ).

<sup>b</sup>Significant differences were observed between AT compared with BM and UCB ( $p < .05$ ).

Abbreviations: AT, adipose tissue; BM, bone marrow; UCB, umbilical cord blood.

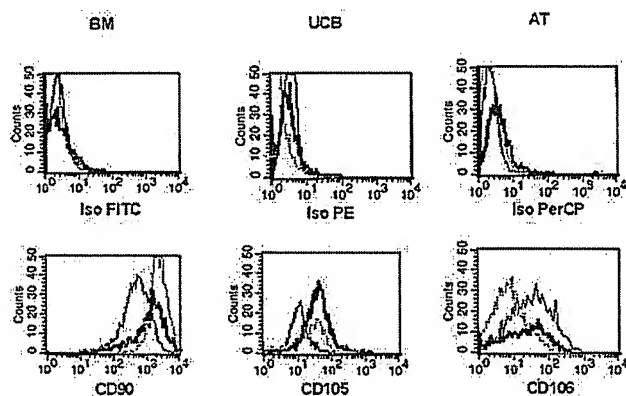
donors at passages 2–5 were examined by flow cytometry. Each sample was analyzed at one distinct passage (Table 2).

MSCs derived from all three sources displayed no expression of hematopoietic markers (CD14, CD34, CD45), of the stem cell marker CD133, or the marker for endothelial cells CD144. More than 90% of MSCs derived from the three sources expressed the typical MSC marker proteins CD44, CD73, CD29, and CD90. However, the intensity of expression of CD90 of UCB-derived MSCs was significantly below that of the other tissues (mean fluorescence intensity: UCB,  $738 \pm 715.9$ ; BM,  $1,512.1 \pm 754.9$ ; AT,  $2,200.6 \pm 896.9$ ; UCB compared with BM,  $p = .02$ ; UCB compared with AT,  $p = .001$ ) (Fig. 3). More than 90% of the MSCs derived from all three sources expressed HLA I; however, none of the MSCs expressed HLA II. CD105 was expressed by a significantly lower percentage of UCB-MSCs compared with BM- or AT-MSCs (UCB compared with BM,  $p = .02$ ; UCB compared with AT,  $p = .008$ ) (Fig. 3). Significantly more UCB- and BM-MSCs expressed CD106 than AT-MSCs (UCB compared with AT,  $p = .008$ ; BM compared with AT,  $p = .002$ ) (Fig. 3). The same was observed for the intensity of expression of this molecule; it was significantly higher for UCB- and BM-MSCs compared with AT-MSCs (BM,  $28.37 \pm 21.18$ ; UCB,  $41.90 \pm 31.81$ ; AT,  $8.26 \pm 4.77$ ; AT compared with BM,  $p = .02$ ; AT compared with UCB,  $p = .001$ ) (Fig. 3).

### DISCUSSION

We compared MSCs from BM and two alternative sources, namely UCB and AT, concerning basic MSC characteristics. All cells isolated from these three sources exhibited typical MSC characteristics: a fibroblastoid morphology, the formation of CFU-F, a multipotential differentiation capability, and the expression of a typical set of surface proteins. Whereas





**Figure 3.** Immunophenotype analysis of mesenchymal stem cells (MSCs) from BM (broken line), UCB (heavy line), and AT (light line). MSCs of nine BM donors at passages 0–7, 13 UCB-fibroblastoid colony-forming units at passages 3–5, and nine AT donors at passages 2–5 were trypsinized, labeled with antibodies against the indicated antigens, and analyzed by flow cytometry. Only representative examples featuring significant different antigen expression profiles are shown. Abbreviations: AT, adipose tissue; BM, bone marrow; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCP, Peridium-chlorophyll protein complex; UCB, umbilical cord blood.

MSCs derived from the three sources expressed classic MSC marker proteins, but lacked hematopoietic and endothelial markers, we observed significant differences concerning the expression of CD90, CD105, and CD106. These molecules are described to be associated with hematopoiesis and cell migration [18–20]. It needs to be further investigated whether these molecules are functionally important for stroma and homing capacities. In a first approach, we created a comprehensive protein expression profile of undifferentiated UCB-MSCs, which will be extended to BM- and AT-MSCs and then correlated to functional properties [21].

Since the relevance of the observed differences of marker expression has not been properly investigated yet, differences concerning differentiation capacity seem to be more relevant for MSC quality at present. We demonstrated a multilineage differentiation capacity for BM- and AT-MSCs. Interestingly, UCB-MSCs could not be differentiated toward the adipogenic lineage, which was not related to the CFU-F origin. Actually, there are conflicting data concerning the adipogenic differentiation capacity of UCB-MSCs [9–12, 22, 23]. Nevertheless, we assume that UCB-MSCs are less sensitive toward the adipogenic differentiation (supported by results of Chang et al. [22]) which might be related to the ontogenetic age of these cells. This is further supported by the fact that adipocytes reside in adult human BM and AT but are absent in fetal BM and by the observation of an increased adipogenesis correlated with age [24]. Further comparative genomic or proteomic approaches are needed to assess the susceptibility toward adipogenesis of MSCs.

None of our UCB-MSCs showed adipogenic differentiation capacity, but all differentiated into both the chondro- and osteogenic lineages. In contrast, a tri-potential differentiation capacity was observed for most AT samples but only for a few BM samples. One sample each of BM and AT was observed to

undergo only the chondrogenic pathway. In accordance with this, a hierarchical or even restricted differentiation potential of MSCs has been reported [1, 13, 25].

In our study, investigations were limited to the mesodermal differentiation capacity. Based on recent reports, however, the spectrum of differentiation of MSCs does not seem to be restricted to this lineage. MSCs derived from all three tissues have been shown to differentiate into further mesodermal lineages and into endo- and ectodermal lineages as well [10–13, 26–33]. Comparative experiments need to be performed to assess responsiveness toward cardiomyogenic, endothelial, hepatic, neuronal, and pancreatic differentiation.

A high impact on clinical exploitation might be related to the abundance and expansion capacity of MSCs. Based on our results, both BM and AT are reliable sources for isolating and expanding MSCs in autologous settings since all preparations gave rise to MSCs. UCB, in contrast, had an isolation efficacy of a maximum of 63% [8]. We attribute these differences to the fact that MSCs are circulating in the prenatal organism and are residing in tissues of the adult [9]. Despite the low frequency of UCB-MSCs, the expansion potential was highest compared with other cell sources. Considering clinical applications, the resulting cell numbers may be similar to both BM and AT, which can be obtained at higher frequencies. One argument against AT might be the limited availability in some patients. However, we believe that due to the high frequency of AT-MSCs, also small fat reservoirs might be sufficient for MSC isolation. BM has been the main source for clinical application of MSCs, such as the treatment of osteogenesis imperfecta, graft versus host disease, and acute myocardial infarction [34–36]. As the number, frequency, and differentiation capacity of BM-MSCs correlate negatively with age, they could be clinically inefficient when derived from elderly patients. In that case, an allogeneic approach would be required. In case a matching donor is required, BM or AT from HLA identical siblings, haplo-identical relatives, or HLA-screened donors might be best choice. Speculating on a “off-the-shelf” product requiring mass production, AT might be a solid starting basis due to the abundance, relatively easy harvest, and high MSCs frequency.

Transplantation of MSCs is currently a highly experimental procedure, resembling the early beginnings of hematopoietic stem cell transplantation. In the latter, BM has been replaced gradually by peripheral blood progenitor cells and umbilical cord blood. Also, in the field of MSCs, alternative sources are intensely investigated, and one day these new sources may replace BM. Taking into account all the advantages and disadvantages of the three sources discussed above, depending on the therapeutic indication, the clinical applications may be based on differentiation capacity, but more likely on the abundance, frequency, and expansion potential of the cells.

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## DISCLOSURES

The authors indicate no potential conflicts of interest.

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